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Polyamides containing α-aminoacids and hydrophilic oxyethylene groups along the chain

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Summary

Biocompatible and potentially biodegradable polyamides (PAs) containing in the chain both peptide bonds and hydrophilic triethyleneoxide segments have been prepared by interfacial polycondensation of sebacoyl dichloride with amide-diamines derived from the 4,7,10-trioxa-1, 13-tridecanediamine and α-aminoacids such as glycine, L-valine and L-phenylalanine. These PAs exhibit moderate inherent viscosity values and show limited solubilities in $CHCl_3$, DMF and DMSO. ^{1}H-NMR and FTIR spectroscopy analysis confirmed the expected structures. DSC and X-rays diffraction spectra indicated crystallinity degrees from 19 to 31%. The melting temperatures range between 135-238 °C. Liquid water absorption measurements indicate a high equilibrium weight-uptake when a glycine residue is present in the amide-diamine moiety. *In vitro* tests carried out using cultures of human fibroblasts showed the biocompatibility of the prepared PAs.

Introduction

Many efforts have been recently devoted to the investigation and preparation of biocompatible, biodegradable synthetic polymers designed for applications in medicine for either the fabrication of biodegradable devices or as drug delivery systems (1). Many of them consist of condensation polymers having incorporated peptide linkages susceptible to enzymatic cleavage. Polyamides (PAs) containing aminoacid residues such as L-leucine, L-alanine and L-phenylalanine have been reported as biodegradable materials (2,3). Goncalves *et al*. prepared polyamides, and polyureas containing L-leucine and L-tyrosine residues in the chain (4). Polyesteramides derived from α-aminoacids and α-hydroxyacids, the polydepsipeptides, have been also investigated as biodegradable polymers (5,6). Furthermore, an appropriate choice of the nature, number and sequence of α-aminoacids, as well as a balance of hydrophilic and hydrophobic characteristics of the other constituents, makes these polymers susceptible to enzymatic cleavage of the peptide bonds by specific enzymes (7-9). We have previously described the synthesis, the characterization and the biocompatibility of aliphatic PAs derived from C_{10} and C_{14} dicarboxylic acids and amide-diamines derived from 1,6-hexanediamine or 1,12-dodecanediamine and L-phenylalanine, L-valyl-L-phenylalanine or L-phenylalnyl-L-valine residues (10,11). In the present paper we report on the synthesis and characterization of hydrophilic

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polyamides obtained from sebacic acid and amide-diamines derived from 4,7,10-trioxa-1,13-tridecanediamine, a diamine containing the hydrophilic triethyleneoxide group, and aminoacids such as glycine (Gly), L-valine (L-Val) and L-phenylalanine (L-Phe).

Experimental

Materials and techniques

The solvents were purified according to standard procedures. Z-Glycine (Z-Gly), Z-L-valine (Z-Val), Z-L-phenylalanine (Z-Phe), isobuthylchloroformate (i-BCCl), methanol and chloroform, from Fluka, were used as received. Sebacoyl chloride and 4,7,10-trioxa-1,13-tridecanediamine (**1**) (Fluka) were distilled under vacuum. IR analysis was performed using a Bruker IFS 66 FTIR spectrophotometer. Bidimensional ^{1}H-NMR spectra were recorded at 25 °C using a Bruker DRX 400 instrument and chloroform-*d* ($CDCl_3$) or dimethylsulfoxide-d_6 (DMSO-d_6) as a solvent. The thermal data were obtained using a Mettler TA-3000 differential scanning calorimeter (DSC) under nitrogen from 50 °C to 250/300 °C at a rate of 10 °C/min. The inherent viscosities were measured at 25 °C in *m*-cresol (c = 0.5 g/dL). The purity of the products was checked by analytical RP-HPLC on a Varian 3000 LC Star System. Wide-angle X-rays diffraction (WAXD) patterns were recorded on a Philips diffractometer equipped with a continuous scan attachment and a proportional counter, using Ni-filtered Cu-Kα. radiation (1,5418 Å). The mass spectra were obtained using the fast bombardment (f.a.b.) analysis carried out on a ZAB 2SE double focusing mass spectrometer (Micromass, UK) equipped with a caesium gun operating at 25 kV (2μA). All mass values are reported as monoisotopic masses. The water absorption was determined at 20°C by immersion in liquid water for different times weighted films (60-80 mg) prepared by solution casting. Upon removal, the samples were blotted on filter paper to remove the excess water from the surface and weighted in closed bottles. This procedure was repeated to constant weight. The biocompatibility was tested using an *in vitro* model. Five samples of each PA were stuck by means of a biocompatible silicone on the bottom of 12-multiwells and a small area was left uncovered in order to directly observe the cells by an inverted microscope. The bottom of polystyrene wells was used as control sample. Before plating the fibroblasts, the materials in the wells were extensively washed with a saline buffered solution containing penicillin (1000 units/mL) and streptomycin (1 mg/mL). The multiwells were then kept in the presence of complete culture medium for at least 48 h at 37°C in a 5% CO_2 humidified atmosphere. 30,000 fibroblasts were seeded *per* each well. 24 h after the cell plating, the cells were observed and evaluated in terms of morphology and adhesion. The MTT test (12) was carried out measuring spectrophotometrically at 570 nm the amount of formazan produced by reduction of the tetrazolium ring of MTT by the living cells.

Synthesis of amide-diamines

a) 1,13-di(L-phenylalaninamido)-4,7,10-trioxatridecane **(2)**

The synthesis and characterization of **2** has been previously reported (10). The m.s. gave a peak for the quasi-molecular ion (MH^+) at m/z at 515.

b) 1,13-di(L-phenylalanylglycinamido)-4,7,10-trioxatridecane **(3)**

i-BCCl (19.8 mL, 150 mmol) was added dropwise at -15 °C under stirring, to 430 mL of a chloroform solution containing Z-Gly (34.4 g, 150 mmol) and triethylamine (21.9 mL, 150

mmol) contained in a two-necked flask equipped with inlet for nitrogen and a dropping funnel. After stirring for 20 min, a solution of **1** (16.6 g, 75.5 mmol) in chloroform (150 mL) was added dropwise and the mixture was stirred overnight at room temperature. The $CHCl_3$ solution was washed with citric acid and $NaHCO_3$ solutions and water and finally dried over anhydrous Na_2SO_4. By removing the solvent at reduced pressure, 1,13-di-(Z-glycinamide)-4,7,10-trioxatridecane (**I)** was recovered as a yellow waxy solid (yield 87%) which was purified by dissolution in acetone and precipitation with ethyl ether (purity > 99%). **I** (6.55 g, 10.8 mmol), dissolved in methanol (430 mL), was treated with hydrogen in the presence of 10% Pd on charcoal (1.15 g) according to reported procedures (10) yielding 3.37 g of 1,13-di(glycinamido)-4,7,10-trioxatridecane (**Ia**) as a viscous colorless oil (yield 93%). ^{1}H-NMR: δ = 3.39 (α, 4H, q); δ = 1.8 (ß, 4H, m); δ = 3.56 (γ, 4H, t); δ = 3.62 (ε, 8H, m); δ = 3.33 (λ, 4H, s); δ = 7.52 (NH, 2H, s); δ = 1.64 (NH_2, 4H, s).

3 was obtained following a similar procedure. i-BCCl (2.00 mL, 15.2 mmol) was reacted with Z-Phe (4.55 g, 15.2 mmol) and triethylamine (2.20 mL, 15.2 mmol) dissolved in 44 mL of chloroform. Successively, 17 mL of chloroform containing **Ia** (1.96 g, 5.88 mmol), were added dropwise at -15 °C to the reaction mixture. An analogous treatment to that used for **I** gave 4.29 g of 1,13-di-(Z-L-phenylalanylglycinamido)-4,7,10-trioxatridecane (**II**) as a white solid (purity 98%). Hydrogenolysis of **II** gave 2.06 g of **3** (69% yield, purity ≥ 95%) as a colorless oil. ^{1}H-NMR: δ = 3.38 (α; 4H,q), δ = 1.78 (ß;4H, m), δ = 3.56 (γ; 4H, t), δ = 3.6 ÷ 3.7(ε; 8H, m), δ = 3.9 (λ;4H,q), δ =7.95 (NH; 2H, s); δ = 1.6 (NH_2; 4H, s), δ 7.15 ÷ 7.40 (Φ; 10H, m), δ ~ 4 (CH, 2H); δ = 6.9 (NH'; 2H, m), δ = 2.7; 3.2 (λ'; 4H, q). The m.s. gave a peak for the quasi-molecular ion (MH^+) at m/z 629.

c) 1,13-di(L-phenylalanyl-L-valylglycinamido)-4,7,10-trioxatridecane **(4)**

1,13-Di(L-valylglycinamido)-4,7,10-trioxatridecane (**III**a) was obtained as colorless oil from **Ia** and Z-Val following the procedure used for **3** (yield 70%); **(4**) was obtained as glassy solid from **III**a and Z-Phe and subsequent hydrogenolysis (yield 94%, purity ≥ 95%). ^{1}H-NMR: δ = 3.34 (α, 4H, m); δ = 1.76 (ß, 4H, m); δ = 3.53 (γ, 4H, t); δ = 3.55 ÷ 3.65 (ε, 8H, m); δ = 3.92 (λ', 4H, 2q); δ = 7.54 (NH, 2M, t); δ = 8.02 (NH', 2H, s); δ = 3.70 (CH', 2H, m); δ = 2.18 (CH'', 2H, m); δ = 0.92; 0.96 (CH_3, 12H, 2d); δ ~ 7.2 (NH'', 2H); δ = 4.23 (CH, 2H, t); δ = 2.72; 3.24 (λ, 4H, 2q); δ = 7.20 ÷ 7.36 (Φ, 10H, m); δ = 1.92 (NH_2, 4H, s). The m.s. gave a peak for the quasi-molecular ion (MH^+) at m/z 828.

d) 1,3-di(L-phenylalanyl-L-valinamido)-4,7,10-troxatridecane (**5***)*

1,13-di(L-valinamido)-4,7,10-trioxatridecane, (**IVa**) was obtained as for **Ia** and the structure was confirmed by ^{1}H-NMR analysis. **5** was obtained as a colorless oil from **IVa** and Z-Phe following the procedure used to prepare **3** (yield 87%, purity ≥ 95%). ^{1}H-NMR: δ = 3.40 (α, 4H, q); δ = 1.80 (ß, 4H, m); δ = 3.60 (γ, 4H, t); δ = 3.66, (ε, 8H, m); δ = 2.75 ÷ 3.25 (CH_2, 4H 2q); δ = 4.24 (CH, 2H, t); δ = 7.29 (Φ, 10H, m); δ = 7.93 (NH, 2H, t); δ = 3.45 (CH', 2H, m); δ = 6.98 (NH', 2H, m); δ = 2.17 (CH'', 2H, m); δ = 0.95 (CH_3, 12H, 2d); δ = 1.71 (NH_2, 4H,s). The m.s. gave a peak for the quasi-molecular ion (MH^+) at m/z 713.

Polymers

All polyamides were prepared by low temperature polycondensation using the stirred interfacial technique. As a typical procedure, we report the synthesis of poly [1,13-di(L-phenylalanylglycinamido)-4,7,10-trioxatridecanesebacamide] (SEB 3 of Table 1).

Freshly distilled sebacoyl dichloride (1.11 g, 4.64 mmol) dissolved in 24 mL of anhydrous chloroform/n-hexane mixture (1:1 by vol) was quickly added under stirring to 2.92 g (4.64 mmol) of **3** and 0.94 g (16.7 mmol) of KOH dissolved in 120 mL of water. The stirring was continued for 8 min. The white fibrous polymer formed was filtered, repeatedly washed with distilled water before being finally dried at 60 °C under vacuum (yield 93%); η_{inh} = 0.50 dL/g (*m*-cresol, 25 °C, *c* = 0.5 g/dL); m.p.= 126 °C, ΔH_m = 25.6 J/g; IR: 3060 (m), 3339 (s), 2926 (m), 2860 (m), 1654 (s), 1560 (m), 1098 (s) cm^{-1} ; ^{1}H-NMR: δ = 8.32 (NH', 2H,t), δ= 8.16 (NH Phe, 2H,d), δ = 7.78 (NH, 2H,t), δ = 7.20 ÷ 7.40 (ϕ, 10H, m), δ = 3.64 ÷ 3.84 (λ, 4H, 2q), δ = 3.18 (α, 4H,q), δ = 3.53 ÷ 3.63 (ε, 8H, m), δ = 3.48 (γ, 4H, t), δ = 2.86; 3.12 (λ', 4H, 2q), δ = 1.72 (ß, 4H,m); sebacic acid residue: δ = 2.12 (αCH_2,4H, t), δ = 1.44 (ßCH_2,4H,m), δ = 1.10÷1.26 (other CH_2).

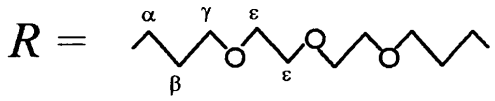
Results and Discussion

Amide-diamines

Amide-diamines containing the hydrophilic triethyleneoxide segment and enzymatically degradable peptide bonds have been prepared from 4,7,10-trioxa-1,13-tridecanediamine, **1**, and α-aminoacids. L-Phe, alone or together with Gly, L-Val or with the Gly-L-Val dipeptide, was used to preform peptide bonds susceptible to cleavage by α-chymotrypsin. These amide-diamines were obtained by means of sequential additions of aminoacid residues to the -NH_2 end groups of **1** according to the well known "mixed anhydride" procedure(13) involving Z-blocked aminoacids. Careful purification of the Z derivatives by precipitation with ethyl ether from an acetone solution gave pure products as tested by means of the hplc technique. Subsequent hydrogenolysis of the Z group in methanol, in the presence of C/Pd, gave pure amide-diamines (purity ≥ 95%) as oils or glassy solids which were soluble in chloroform, dichloromethane, ethanol, methanol and water. The structural features of the prepared diamines **2-5** are shown in Figure 1. The chemical structure was determined by FTIR and ^{1}H-NMR spectroscopy. The FTIR spectra show characteristic absorptions of the amide group at 3360-3300 (s), 1655-1650 (vs) and 1535-1525 (s) cm^{-1}, of aromatic rings at 3060-3020 (w) cm^{-1} and an absorption at 1108(s) cm^{-1} attributable to the C-O-C group. The ^{1}H-NMR data are in full agreement with the expected structures.

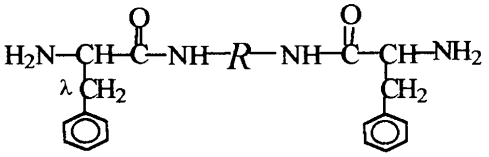
Polymers

The polyamides were obtained from the diamines **1-5** and sebacoyl chloride. Those derived from monomers **2-5** are characterized by the presence in the chain of both a triethyleneoxide segment and a L-Phe residue. The former provides hydrophilicity and chain flexibility, thus favoring the interaction between the polymer chain and the enzyme, whereas the latter is involved in peptide bonds susceptible to attack by α-chymotrypsin. Moreover, an enhancement of the biodegradation rate is expected when the enzymatically cleavable linkage is incorporated in an appropriate sequence of 2-4 aminoacids units (8). A polyamide derived from diamine **1**, which does not contain aminoacid residues, SEB 1, was prepared for the sake of comparison in order to investigate the influence of the nature, the number and the sequence of aminoacids on the properties of this class of polyamides. The stirred low temperature interfacial polycondensation technique was used to obtain the polymers. This procedure was suggested by the fair solubility of the diamines in water. A $CHCl_3$/*n*-hexane (1:1 by vol) solution was used as a solvent of the sebacoyl chloride. The

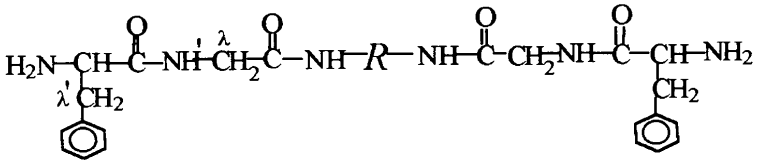


$H_2N—R—NH_2$

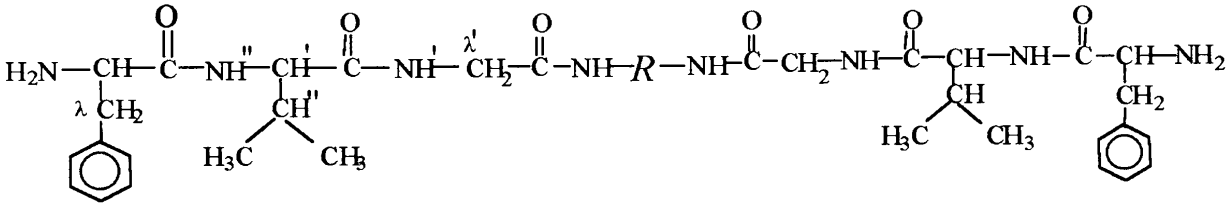
4,7,10-Trioxa-1,13-tridecanediamine **(1)**



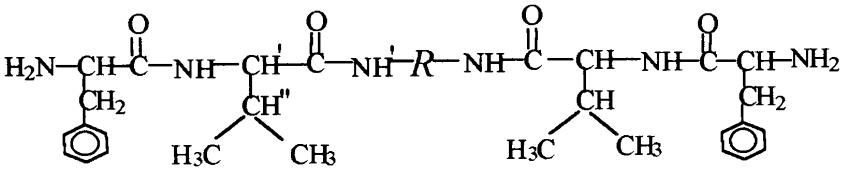
1,13-di(L-phenylalaninamido)-4,7,10-trioxatridecane **(2)**



1,13-di(L-phenylalanylglycinamido)-4,7,10-trioxatridecane **(3)**



1,13-di(L-phenylalanyl-L-valylglycinamido)-4,7,10-trioxatridecane **(4)**



1,13-di(L-phenylalanyl-L-valinamido)-4,7,10-trioxatridecane **(5)**

Figure 1. Amide-diamines used in the preparation of polyamides. The Greek letters refer to the ^{1}H-NMR spectra.

presence of *n*-hexane favors the precipitation of the resulting polymers reducing their solubility in the organic phase. White fibrous polymers were recovered with high yields (70-93%). The prepared polyamides are reported in Table I together with their thermal and solubility data. The inherent viscosity values range between 0.50-1.02 dL/g and indicate moderate degrees of polymerization. They are soluble in typical polyamides solvents such as formic acid and *m*-cresol and also, to some extent, in chloroform and polar aprotic solvents such as dimethylsulfoxide (DMSO) and dimethylformamide (DMF). Flexible films were obtained by solution casting. The FTIR analysis of SEB 2-5 polymers shows characteristic amide bands at 3300-3340 (s), 1638-1654 (s), 1545-1560 (m) cm^{-1} and absorptions at 1098-1110 (s) cm^{-1} (C-O-C stretching), at 2926 (m) and 2960 (m) cm^{-1}

(-CH_2-stretching) and at 3060 (w) cm^{-1} (=CH- aromatic stretching vibration). The thermal behavior, which was investigated by DSC technique, indicates a semicrystalline character for all polyamides. Their DSC traces show, in fact, a second order transition in the 36-66°C temperature range and a single well defined endotherm in the case of SEB 1, SEB 3, SEB 4 and SEB 5, while two broad melting endotherm are present in the thermograrn of SEB 2. The latter polymer also shows a ΔH_m value lower than that of SEB 1, indicating a more difficult crystallization caused by the benzyl group of the L-Phe residue. Such an effect, however, is reduced when L-Phe is part of a sequence of two or three aminoacids, as in SEB 3-5 polymers. It is worth noting that indications of' thermal decomposition for SEB 1-5 were not found up to 260-270 °C, in either the DSC traces or thermogravimetric analysis experiments. The combination of low T_m and high decomposition temperatures allows the formation of films by melt casting. X-rays diffraction diagrams of polymer films obtained by solution casting are consistent with the DSC results showing crystalline patterns characterized by two strong diffraction maxima at 20.4 and 22.5 of 2Θ for SEB 1 and by a single strong reflection at 19.3, 19.7, 19.4 and 19.3 of 2Θ in the case of SEB 2,3,4,5 polymers, respectively. A second broad peak at higher values of 2Θ is observable as a shoulder of the main peak in the spectrum of SEB 2; its intensity reduces markedly by increasing the number of the aminoacids in the chain repeat unit. The degree of crystallinity, evaluated by subtracting the contribution of the amorphous part from the total scattering intensity, are reported in Table 1. The amount of the crystalline fraction in the aminoacid containing polyamides SEB 2-5 is always lower than that of SEB 1 (see also the ΔH_m values). On the other hand, the crystallinity degree increases with increasing the number of the aminoacid residues in the chain repeat unit, thus suggesting that the peptide segments contribute to the crystallinity of these materials. The hydrophilicity of the SEB 1-5 polyamides was investigated by determining the equilibrium concentration of absorbed liquid water at 20°C. SEB 1 polyamide showed a high weight-uptake after immersion for 14 days in water (11% by wt), whereas a sample of the hydrophobic poly(1,12-dodecametilenesebacamide), which was prepared for the sake of comparison, showed less than 1% of absorbed water under the same experimental conditions. As far as SEB 2-5 behavior is concerned, the content of absorbed water is strongly dependent on the nature of aminoacid residues. In fact, in the case of Gly containing polymers SEB 3 and SEB 4 we found amounts of absorbed water higher than those found for SEB 1 (19 and 16% by

Table 1. Characterization of polyamides obtained from sebacoyl chloride and diamines **1-5**

Polymer code	Diamine	Yield %	η_{inh} dl/g	T_m (°C)	ΔH_m (J/g)	T_g (°C)	Water uptake [a]	cryst [b] %	Solubility [c] $CHCl_3$	DMSO	DMF
SEB 1	**1**	70	1.02	135	70	40	11	38	+	–	sw
SEB 2	**2**	70	1.02	148 174	18	44	6	19	+	+	+
SEB 3	**3**	93	0.50	126	26	36	19	26	sw	++	++
SEB 4	**4**	87	0.54	213	39	58	16	31	sw	++	sw
SEB 5	**5**	89	0.54	238	60	66	1	23	+	sw	sw

a) Equilibrium concentration of absorbed water (% by wt) at 20°C; b) degree of crystallinity obtained from X-rays diffraction patterns; c) ++ soluble; + soluble by heating; – insoluble; sw swelling.

wt, respectively), while in the case of SEB 2 and SEB 5 polyamides containing only aminoacids having hydrophobic side chains, the water absorption is drastically reduced with respect to that of SEB 1 (6 and 1% by wt, respectively). In the X-rays diffraction patterns of the water saturated SEB polymers, the position of the diffraction maxima are unchanged with respect to those of the corresponding dry samples. It is, therefore, reasonable to assume that the water absorption occurs preferably in the amorphous phase. The whole of the above results, however, does not seem to indicate a relationship between crystallinity and the amount of absorbed water.

Biocompatibility

The biocompatibility was investigated employing an *in vitro* model according to the direct contact method in which the cells are directly cultured on the material. Primary cultures of human fibroblasts were used as cellular model. Regardless of the chemical structure, no difference was observable in the morphology of the cells. On the contrary cell adhesion was affected by the material features. Indeed, the adhesion efficiency (data not shown) was high as the control (90%) for SEB 1, while the lowest value (52%) was found for SEB 3; values in this range (61% and 74%) were detected in the case of SEB 2 and SEB 5, respectively. The cell growth and viability was assessed by the MTT test six days after the cell plating. The results obtained, shown in Figure 2, are consistent with the initial adhesion values, thus confirming a decreased cell anchorage capacity, rather than a lower growth rate of fibroblasts cultured on SEB 2, SEB 3 and SEB 5. In conclusion these data demonstrate the biocompatibility of the investigated polyamides.

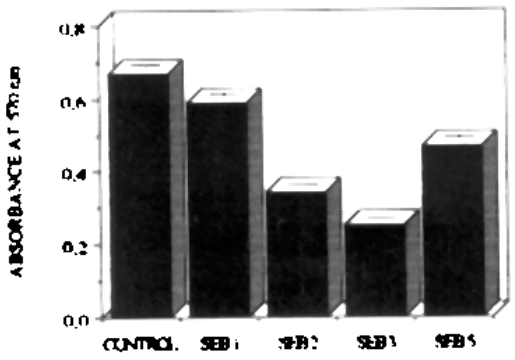


Figure 2. MTT test on fibroblasts after 6 days from plating onto different polymers

Conclusions

A series of novel amide-diamines containing oxyethylene groups and L-Phe or L-Phe based di- or tri-peptides can be successfully used to prepare semicrystalline biocompatible polyamides of moderate polymerization degree. The melting temperature and the amount of absorbed water of the PAs depend on the nature and the number of aminoacid residues in the chain repeat unit. The presence of both peptide bonds targeted for enzymatic attack and flexible, hydrophilic triethyleneoxide sequences makes these polymers promising for biomedical applications as biodegradable materials.

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